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Epigenetic Biomarkers in Diffuse  
Large B-Cell Lymphoma  
Sofia Paupério Paulino

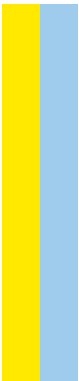
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Sofia Margarida de Castro Paupério e Silva Paulino





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## **EPIGENETIC BIOMARKERS IN DIFFUSE LARGE B-CELL LYMPHOMA**

Dissertação de Mestrado em Oncologia apresentada ao  
Instituto de Ciências Biomédicas Abel Salazar da  
Universidade do Porto – Especialização Oncologia Molecular

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## RESUMO

O Linfoma difuso de grandes células B (LDGCB) é o subtipo mais comum dos Linfomas Não-Hodgkin, correspondendo a cerca de 30% a 40% de novos casos e a mais de 80% dos linfomas agressivos. Devido à sua heterogeneidade clínica, morfológica, imunológica e genética, torna-se pertinente, aquando do diagnóstico, identificar os doentes que irão beneficiar da administração de um tratamento mais agressivo. O sistema de classificação segundo o estadio Ann Arbour, não é, por si só, suficiente, sendo necessário a utilização do Índice Internacional de Prognóstico como ferramenta mais completa. No entanto, têm surgido novos marcadores de prognóstico. Assim, torna-se essencial um melhor conhecimento das alterações epigenéticas, que constituem o alvo do presente estudo, para a aplicação de decisões terapêuticas ajustadas e personalizadas na tentativa de obter melhores resultados clínicos

Neste estudo, tivemos por objetivo avaliar a expressão das modificações epigenéticas pós-traducionais das histonas H3K4me3 (trimetilação da lisina 4 da histona H3) e H3K27me3 (trimetilação da lisina 27 da histona H3), correlacionando-as com os parâmetros clínicos e patológicos, determinando o seu potencial como biomarcadores de prognóstico em LDGCB.

Com este propósito, foi realizado um estudo transversal retrospectivo baseado em 155 casos de Linfoma difuso de grandes células B consecutivamente diagnosticados e tratados no Instituto Português de Oncologia do Porto, entre 2008 e 2013, do qual existia material biológico de arquivo disponível para avaliação da imunoexpressão das marcas epigenéticas H3K4me3 e H3K27me3, utilizando um sistema de quantificação baseado em análise de imagem digital para determinar os parâmetros “percentagem de células positivas” e o “Hscore”. Foi utilizada a base de dados do Serviço de Oncohematologia para obtenção dos parâmetros clínicos e patológicos relevantes.

Com base na análise da sobrevivência, o sistema de classificação Ann Arbour agrupado ( $p=0.015$ ), o IPI agrupado ( $p=0.041$ ) e a idade quando o diagnóstico ( $\leq$  ou  $> 60$  anos e  $\leq$  ou  $> 70$  anos,  $p=0.006$  e  $p=0.005$ , respetivamente) foram confirmados como fatores de prognóstico. Na avaliação das marcas epigenéticas H3K4me3 e H3K27me3, não se observou associação com a sobrevivência global nem com a sobrevivência livre de doença, embora se tenha identificado uma associação entre o IPI ( $p=0.41$ ) e a marca epigenética H3K27me3.

A coorte de pacientes estudados é globalmente representativa dos casos de LDGBCB, conforme atestado pela verificação da associação entre diversos parâmetros clínicos e o prognóstico. Contudo, as marcas epigenéticas H3K4me3 e H3K27me3 não revelaram valor prognóstico nesta neoplasia. Fatores como a dimensão da amostra e a ausência de categorização de acordo com a célula de origem poderão contribuir para explicar este resultado negativo. Assim, será necessário incrementar a série e melhor caracterizá-la imunofenotipicamente para concluir mais definitivamente sobre o potencial valor clínico destes biomarcadores epigenéticos.

**Palavras-Chave:** Linfoma não Hodgkin, linfoma difuso de grandes células B, epigenética, biomarcadores, Imunocitoquímica, prognóstico.

## SUMMARY

Diffuse large B-cell lymphoma (DLBCL), a heterogenous category of aggressive lymphoid malignancies, is the most common type of B-cell non-Hodgkin lymphoma, accounting for approximately 30 to 40% of all newly diagnosed cases worldwide and 80% of aggressive lymphomas. The DLBCL lymphomagenesis remains complex and mostly unknown. Prognostic information for most NHL is not accurately provided by the Ann Arbor system and it is of limited value for treatment decisions. International Prognostic Index (IPI) and R-IPI remain the most reliable tools for predicting outcome in patients with DLBCL. Additional prognostic markers are under investigation. Epigenetic changes are now recognized as playing an important and early role in the lymphomagenesis process, contributing to the disease progression.

In this study, we aimed to quantitatively evaluate the expression of the post-translational epigenetic modification of H3K4me3 (trimethylation of lysine 4 on histone H3) and H3K27me3 (trimethylation of lysine 27 of histone 3) in a series of diffuse large B-cell lymphoma through immunoexpression and correlation with clinicopathological parameters, to determine its relevance as prognostic biomarker.

A retrospective transversal study was performed using archival biological material from 155 patients with diffuse large B-cell lymphoma, consecutively diagnosed and treated between 2008 and 2013 at the Portuguese Oncology Institute of Porto. The H3K4me3 and H3K27me3 marks were assessed by immunohistochemistry, using a digital imaging assisted system to determine the % of positively immunostained cells and the Hscore. Relevant clinical and pathological data was extracted from the database of the Department of Oncohematology.

In this study, grouped Ann Arbor stage ( $p=0.015$ ), grouped IPI score ( $p=0.041$ ) and age at the time of diagnosis ( $\leq$  or  $>$  60 years and  $\leq$  or  $>$  70 years,  $p=0.006$  and  $p=0.005$ , respectively) were all significantly associated with prognosis. Quantitative H3K4me3 and H3K27me3 immunoexpression did not associate with overall or disease-specific survival, although a significant association between IPI score and H3K27me3 mark was disclosed ( $p = 0.041$ ).

Our patient cohort is globally representative of DLBCL patients as depicted by the significant associations between established prognostic parameters and patient outcome. Nevertheless, H3K4me3 and H3K27me3 immunoexpression were not shown to display prognostic value in this dataset. This negative result might be partially explained by insufficient sample size and lack of discrimination of DLBCL subtypes according to cell of

origin. Thus larger studies, with increased sample size to provide a more robust statistical analysis and additional immunophenotyping for DLBCL subtyping are required to more conclusively determine whether H3K4me3 and H3K27me3 might be of prognostic value in DLBCL.

**Keywords:** Diffuse large B-cell lymphoma, epigenetics, molecular biomarkers, non-Hodgkin lymphoma, immunohistochemistry, H3K4me3, H3K27me3, prognosis.

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**LIST OF ABBREVIATIONS**

|                 |   |
|-----------------|---|
| <b>ABC</b>      | Activated B-cell like   |
| <b>BCR</b>      | B-cell receptor   |
| <b>CHOP</b>     | Cyclophosphamide, hydroxydaunorubicin, oncovin and prednisone |
| <b>COO</b>      | Cell of origin  |
| <b>CpG</b>      | Cytosine-phosphatidyl-guanine                                 |
| <b>CpGs</b>     | Cytosine-phosphatidyl-guanine islands                         |
| <b>DLBCL</b>    | Diffuse large B-cell lymphoma                                 |
| <b>DNMT</b>     | DNA methyltransferase   |
| <b>ECOG</b>     | Eastern cooperative oncology group                            |
| <b>EUA</b>      | United States of America                                      |
| <b>EZH2</b>     | Enhancer of zeste homolog 2                                   |
| <b>GCB</b>      | Germinal center B-cell like                                   |
| <b>H3K27me3</b> | Trimethylation of lysine 27 on histone H3                     |
| <b>H3K4me3</b>  | Trimethylation of lysine 4 on histone H3                      |
| <b>HHV8</b>     | Human herpes virus 8  |
| <b>HIV</b>      | Human immunodeficiency virus                                  |
| <b>HP</b>       | <i>Helicobacter pylori</i>                                    |
| <b>ICD-O</b>    | International classification of diseases for Oncology         |
| <b>IPI</b>      | International prognostic index                                |
| <b>LDH</b>      | Serum lactate dehydrogenase                                   |
| <b>LH</b>       | Hodgkin's Lymphomas   |
| <b>MLL</b>      | Mixed lineage leukemia  |
| <b>ncRNA</b>    | Non-coding RNA  |
| <b>NK</b>       | Natural Killer  |

|               |  |
|---------------|--|
| <b>NLH</b>    | Non-Hodgkin's Lymphomas  |
| <b>NOS</b>    | Not otherwise specified  |
| <b>OS</b>     | Overall survival   |
| <b>PMBCL</b>  | Primary mediastinal large B-cell lymphoma                                |
| <b>PRC2</b>   | Polycomb repressive complex 2  |
| <b>R-CHOP</b> | Rituximab, cyclophosphamide, hydroxydaunorubicin, oncovin and prednisone |
| <b>R-IPi</b>  | Re-categorized international prognostic index                            |
| <b>WHO</b>    | World Health Organization  |

# **INTRODUCTION**



Lymphoma is the malignant neoplasm from lymphoid system cells. It is predominantly located in the lymph nodes or other extra lymph node location, such as spleen, bone marrow, skin, gastrointestinal tract and other organs <sup>(1,2)</sup>. The World Health Organization (WHO) classifies lymphomas into two major groups, comprising Hodgkin's Lymphomas (LH) and Non-Hodgkin's Lymphomas (NHL). Hodgkin lymphoma and Non-Hodgkin lymphoma can be differentiated pathologically, by its pattern of disease, treatment and prognosis <sup>(2)</sup>.

Non-Hodgkin's Lymphomas are a heterogeneous group of diseases that originate from mature and immature B, T or Natural Killer (NK) cells <sup>(2)</sup>, representing 4% of all cancers, which are more frequent in developed countries, such as United States of America (EUA), Australia and Western Europe <sup>(2,3)</sup> (Figure 1).

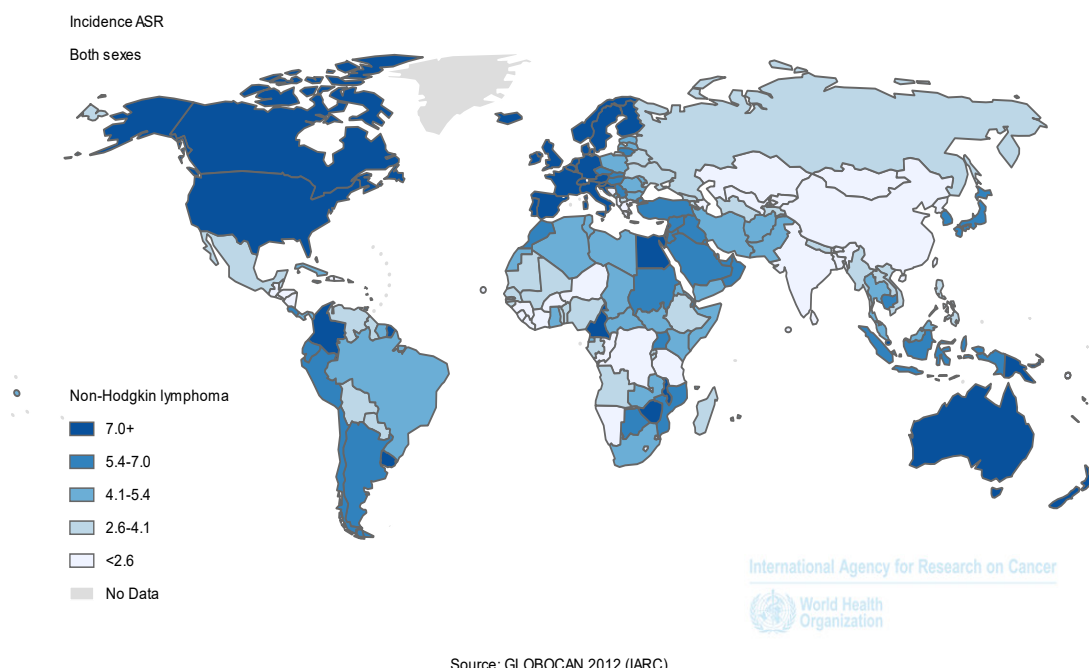
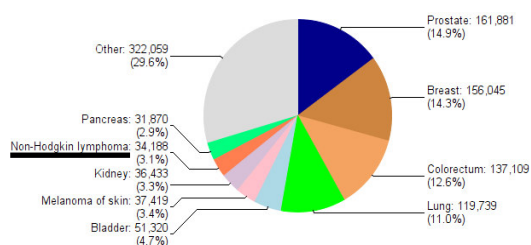


Figure 1- Estimated age-standardized incidence rates of NHL per 100,000, worldwide. Adapted from <sup>(3)</sup>.

In Western Europe, the estimated incidence in 2012 was 34,188 new cases, accounting for 3.1% of all diagnosed cancers (Figure 2) <sup>(3)</sup>. The same trend was reported for Portugal, in which the estimated incidence rate, for the same year, was 1,842 new cases, accounting for 3.7% of all cancers (Figure 2) <sup>(3)</sup>.

InterWestern Europe: Both sexesResearch on Cancer  
 Estimated number of cancer cases, all ages (total: 1088,063)



InterPortugal: Both sexesResearch on Cancer  
 Estimated number of cancer cases, all ages (total: 49,174)

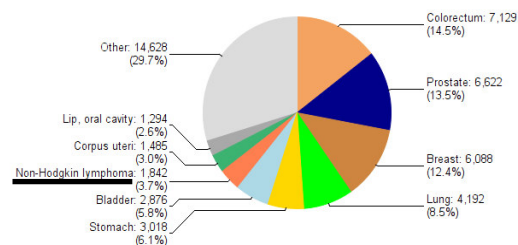


Figure 2 - Estimated incidence of distinct types of cancer in Western Europe and Portugal in 2012, number of newly diagnosed cases and proportion of each cancer comparing to all types of cancer (in both genders, excluding non-melanoma skin cancers). Adapted from <sup>(3)</sup>.

In 2016, the WHO classification of hematopoietic and lymphoid tumours was revised <sup>(4)</sup>. Although alterations are limited compared with the 2008 version, it will include a large set of information published over the last 8 years with significant diagnostic, prognostic and therapeutic implications <sup>(4)</sup>. The updated classification of the mature B-cell neoplasms is depicted in Table 1 and constitutes the worldwide gold standard.

Table 1 – 2016 WHO classification of Mature B-Cell, T and NK neoplasms. Adapted from <sup>(4)</sup>.

| Mature B-cell neoplasm  |
|---|
| Chronic lymphocytic leukemia/small lymphocytic lymphoma   |
| Monoclonal B-cell lymphocytosis*  |
| B-cell prolymphocytic leukemia  |
| Splenic marginal zone lymphoma  |
| Hairy cell leukemia   |
| <i>Splenic B-cell lymphoma/leukemia, unclassifiable</i>   |
| <ul style="list-style-type: none"> <li><i>Splenic diffuse red pulp small B-cell lymphoma</i></li> <li><i>Hairy cell leukemia-variant</i></li> </ul> |
| Lymphoplasmacytic lymphoma  |
| <ul style="list-style-type: none"> <li>Waldenström macroglobulemia</li> </ul>   |
| Monoclonal gammopathy of undetermined significance(MGUS), IgM*  |
| μ heavy-chain disease   |
| γ heavy-chain disease   |
| α heavy-chain disease   |
| Monoclonal gammopathy of undetermined significance(MGUS), IgG/A*  |



**Table 1- (continued)**

|   |
|---|
| Plasma cell myeloma   |
| Solitary plasmacytoma of bone   |
| Extraosseous plasmacytoma   |
| Monoclonal immunoglobulin deposition diseases*  |
| Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)  |
| Nodal marginal zone lymphoma <ul style="list-style-type: none"> <li>• <i>Pediatric nodal marginal zone lymphoma</i></li> </ul>                                |
| Follicular lymphoma <ul style="list-style-type: none"> <li>• In situ follicular lymphoma*</li> <li>• Duodenal-type follicular lymphoma*</li> </ul>            |
| Pediatric-type follicular lymphoma*   |
| <i>Large B-cell lymphoma with IRF4 rearrangement*</i>   |
| Primary cutaneous follicle center lymphoma  |
| Mantle cell lymphoma <ul style="list-style-type: none"> <li>• In situ mantle cell neoplasia*</li> </ul>   |
| Diffuse large B-cell lymphoma (DLBCL), NOS <ul style="list-style-type: none"> <li>• Germinal center B-cell Type*</li> <li>• Activated B-cell type*</li> </ul> |
| T-cell/Histiocyte-rich large B-cell lymphoma  |
| Primary DLBCL of the central nervous system (CNS)   |
| Primary cutaneous DLBCL, leg type   |
| EBV <sup>+</sup> DLBCL, NOS*  |
| EBV <sup>+</sup> <i>mucocutaneous ulcer*</i>  |
| DLBCL associated with chronic inflammation  |
| Lymphomatoid granulomatosis   |
| Primary mediastinal (thymic) large B-cell lymphoma  |
| Intravascular large B-cell lymphoma   |
| ALK <sup>+</sup> large B-cell lymphoma  |
| Plasmablastic lymphoma  |
| Primary effusion lymphoma   |
| HHV8 <sup>+</sup> DLBCL, NOS*   |
| Burkitt lymphoma <ul style="list-style-type: none"> <li>• <i>Burkitt-like lymphoma with 11q aberration*</i></li> </ul>  |
| High-grade B-cell lymphoma, with <i>MYC</i> and <i>BCL2</i> and/or <i>BCL6</i> rearrangements*  |
| High-grade B-cell lymphoma, NOS*  |
| B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma  |

**Table 1- (continued)**

| <b>Mature T and NK neoplasms</b>  |
|---|
| T-cell prolymphocytic leukemia  |
| T-cell large granular lymphocytic leukemia  |
| <i>Chronic lymphoproliferative disorder of NK cells</i>   |
| Aggressive NK-cell leukemia   |
| Systemic EBV <sup>+</sup> T-cell lymphoma of childhood*   |
| Hydroa vacciniforme-like lymphoproliferative disorder*  |
| Adult T-cell leukemia/lymphoma  |
| Extranodal NK/T-cell lymphoma, nasal type   |
| Enteropathy-associated T-cell lymphoma  |
| Monomorphic aptheliotropic intestinal T-cell lymphoma*  |
| <i>Indolent T-cell lymphoproliferative disorder of the GI tract*</i>  |
| Hepatosplenic T-cell lymphoma   |
| Subcutaneous panniculitis-like T-cell lymphoma  |
| Mycosis fungoides   |
| Sézary syndrome   |
| Primary cutaneous CD30 <sup>+</sup> T-cell lymphoproliferative disorders <ul style="list-style-type: none"> <li>• Lymphomatois papulosis</li> <li>• Primary cutaneous anaplastic large cell lymphoma</li> </ul> |
| Primary cutaneous $\gamma\delta$ T-cell lymphoma  |
| <i>Primary cutaneous CD8<sup>+</sup> aggressive epidermotropic cytotoxic T-cell lymphoma</i>  |
| <i>Primary cutaneous acral CD8<sup>+</sup> T-cell lymphoma</i>  |
| <i>Primary cutaneous acral CD4<sup>+</sup> small/medium T-cell lymphoproliferative disorder*</i>  |
| Peripheral T-cell lymphoma*   |
| Angioimmunoblastic T-cell lymphoma  |
| <i>Follicular T-cell lymphoma*</i>  |
| <i>Nodal peripheral T-cell lymphoma with TFH phenotype*</i>   |
| Anaplastic large-cell lymphoma, ALK <sup>+</sup>  |
| Anaplastic large-cell lymphoma, ALK <sup>-</sup> *  |
| <i>Breast implant-associated anaplastic large-cell lymphoma*</i>  |

Italics - Provisional entities/ \*Changes from the 2008 classification.

In this Dissertation, the focus will be on a specific type of the NHL, the Diffuse Large B-Cell Lymphomas (DLBCL), which will be characterized in the next sections.

## 1 - DIFFUSE LARGE B-CELL LYMPHOMA

### 1.1 - Epidemiology and Etiology

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous category of aggressive lymphoid malignancies and represents the most common type of B-cell NHL, accounting for approximately 30% to 40% of all newly diagnosed cases worldwide <sup>(5,6,7,8)</sup>. Given the aggressiveness of this disease, more than 50% of patients may be cured by current immune-chemotherapy protocols <sup>(5,9,10)</sup>. The International Classification of Diseases for Oncology (ICD-O) is used to code malignant tumours using a coherent system and ICD-O code for DLBCL is 9680/3 <sup>(1)</sup>. DLBCL lymphoma may affect patients of all ages but are mostly diagnosed in the elderly. The median age is at 60-70 years but it may occur in children and young adults as well <sup>(11,12,13)</sup>. DLBCL is slightly more common in men than in women <sup>(1,2)</sup> with a M/F ratio of 1.7:1<sup>(14)</sup>.

The lymphomagenesis of DLBCL remains complex and mostly unknown. It can arise *de novo*, i.e., as a primary lymphoma but it may also derive from progression or transformation of a less aggressive (clinically indolent) lymphoma. There is a diversity of factors that may be involved on lymphomagenesis such as immunosuppression (significant risk factor), infectious agents (including human immunodeficiency virus - HIV, human herpes virus 8 - HHV8 and *Helicobacter pylori* - HP), toxicity from cytotoxic drugs and tumour biology <sup>(1, 2, 15)</sup>.

### 1.2 - Sites of involvement and clinical features.

Patients may present nodal and/or extranodal disease. The most common extranodal sites are gastrointestinal tract, bone, head and neck, liver, kidney and thyroid <sup>(1, 16)</sup>. Nearly 10% to 25% of patients with DLBCL disease disclose bone marrow involvement <sup>(17, 18, 19)</sup>.

The clinical manifestations of DLBCL are diverse and depend on the site of disease involvement. Patients usually present with rapidly growing masses, causing some symptoms, but usually patients are asymptomatic <sup>(1)</sup>.

### 1.3 - Morphology

DLBCL is characterized by B lymphoid cells with “*nuclear size equal to or exceeding normal macrophage nuclei or more than twice the size of a normal lymphocyte, that has a diffuse growth pattern*” <sup>(1)</sup> (Figure 3).

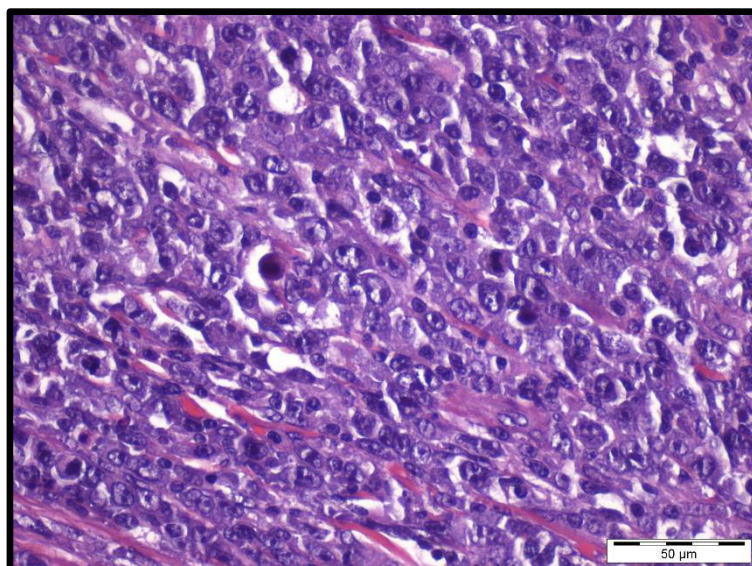


Figure 3 – Diffuse large B-cell lymphoma typical cells (Hematoxylin and Eosin stain-H&E, case 286. Source: Department of Pathology, IPO PORTO.

It is divided into morphological variants, molecular and immunophenotypically subgroups and different disease entities <sup>(1)</sup>. The heterogeneity of some cases make it impossible sometimes to include them on the previously defined subgroups, and these are classified as DLBCL, not otherwise specified (NOS). Thus, DLBCL, NOS includes all DLBCL cases that do not fit in the specific subgroups mentioned above <sup>(1)</sup>.

#### 1.3.1 - Morphological variants

There are three morphological variants recognized <sup>(1)</sup>:

- Centroblastic variant – predominantly composed of centroblasts which “*are medium-sized to large lymphoid cells with oval to round, vesicular nuclei containing fine chromatin*”. It may contain two to four nuclear membrane-bound nucleoli. The cytoplasm is frequently scanty and amphophilic to basophilic. Frequently the tumour is polymorphic with an admixture of centroblasts and immunoblasts (<90%). This is the most common variant.

- Immunoblastic variant – in which “*greater than 90% of the cells are immunoblasts with a single centrally located nucleolus and an appreciable amount of basophilic cytoplasm*”.
- Anaplastic variant - characterized by “*large to very large round, oval or polygonal cells with bizarre pleomorphic nuclei*”.

### 1.3.2 - Molecular subgroups

Based on similarities to the cell of origin (COO), DLBCL can be subdivided into three subtypes: the germinal center B-cell like (GCB) DLBCL, activated B-cell-like (ABC) DLBCL and primary mediastinal large B-cell lymphoma (PMBCL) <sup>(1,5,6,9)</sup>. This classification includes gene alterations and drug sensitivities.

GCB cells are produced from mature B cells in lymphoid organs that have been activated by interactions with an antigen and T-helper cells. The differentiation on GCB cells activates somatic hypermutation, leading to diversity in the immunoglobulin gene regions <sup>(5,20)</sup>. ABC DLBCL has a gene expression feature identical to plasma cells, arise from post germinal center B cells that are arrested during plasmocytic differentiation <sup>(5,20)</sup>. Primary mediastinal large B-cell lymphoma presents with mediastinal lymphadenopathy and has some molecular genetic similarities with Hodgkin lymphoma <sup>(5, 20)</sup>.

By next-generation sequencing techniques it was found that alterations of chromatin remodelling genes are predominant in GCB subtype whereas gene mutations of B-cell receptor (BCR) signalling and NF-kB pathway are frequent in ABC subtype <sup>(6)</sup>. The ABC DLBCL subgroup has a poorer overall survival compared to GCG DLBCL subgroup <sup>(5,6)</sup>.

### 1.3.3 - Immunophenotypical subgroups

Immunohistochemical staining is used to make an approximation to the cell-of-origin subtype of DLBCL. It allows for the discrimination of GCB from non-GCB subgroups. The Hans algorithm <sup>(9)</sup> which uses the antibodies CD10, BCL6, and IRF4, also called MUM1 <sup>(21,22,23)</sup>, is the most widely used (Figure 4 and 5).

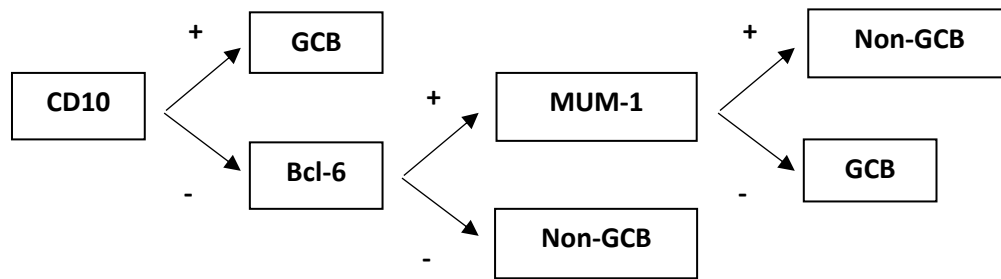


Figure 4 – Hans algorithm based on the immunohistochemical analysis of three markers (CD10, Mum1 and bcl6). Adapted from <sup>(9)</sup>.

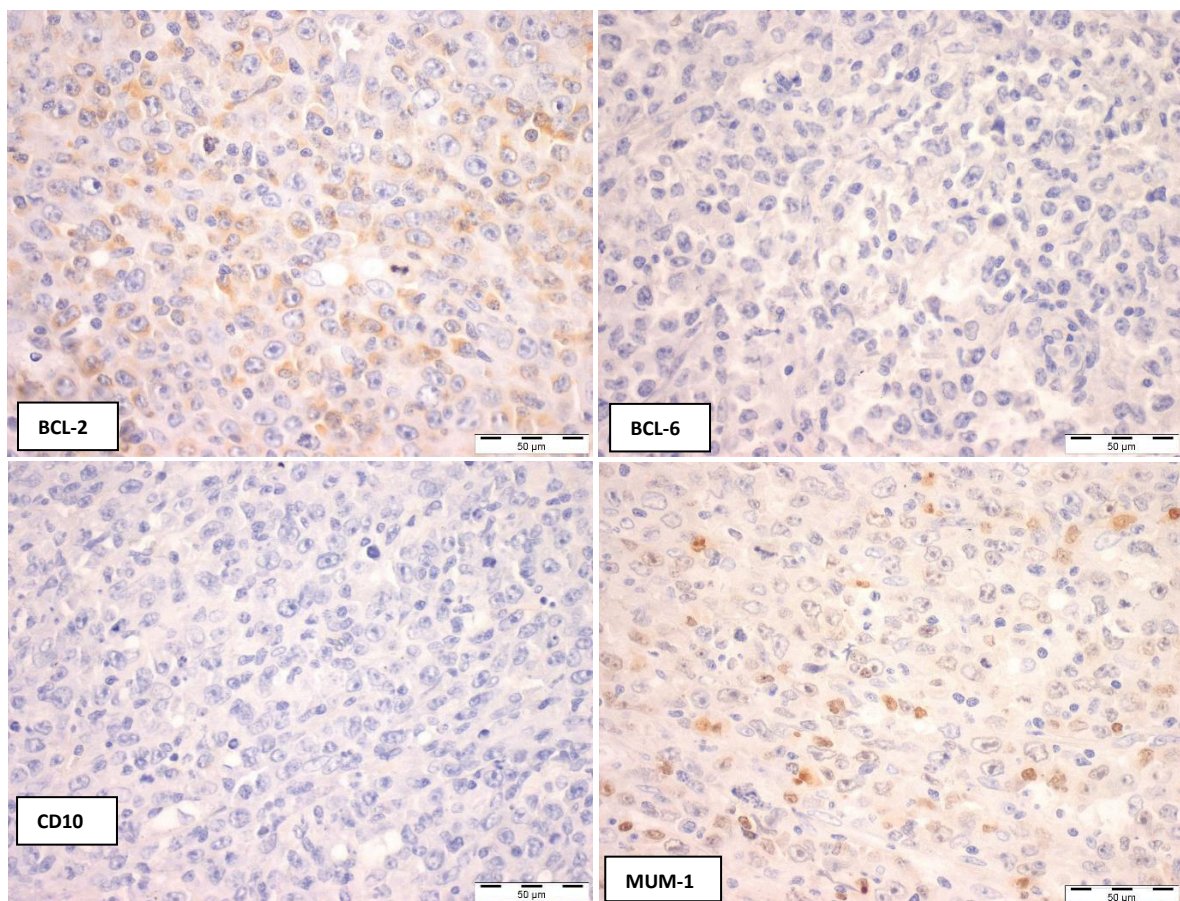


Figure 5 - Example of immunohistochemical staining for DLBCL. Case 286: CD10 -, BCL6-, MUM1 +, a Non-GCB subtype. Source: Department of Pathology, IPO Porto.

#### 1.4 - **Staging**

Choosing the most appropriate therapy involves accurate diagnosis and careful staging evaluation. The first staging concept was developed during the 20<sup>th</sup> century in the United States. Disease heterogeneity was a challenge for development of a uniform staging system. The first staging system was named Ann Arbour Staging System and it was used for Hodgkin lymphomas, but it was later modified and adapted for NHL, providing an anatomic staging <sup>(24)</sup>.

The Ann Arbour Staging System divides patients into four stages, depending on whether it is localized disease, multiples sites of disease on one or the other side of the diaphragm, lymphatic disease on both sides of diaphragm and disseminated extranodal disease <sup>(1, 24)</sup> (Table 2).

**Table 2 – Ann Arbour Classification. Adapted from <sup>(24)</sup>.**

| Stage                      | Features   |
|----------------------------|--|
| <b>I</b>                   | Involvement of a single lymph node region or lymphoid structure (e.g. spleen, thymus, Waldeyer's ring) |
| <b>II</b>                  | Involvement of two or more lymph node regions on the same side of diaphragm                            |
| <b>III</b>                 | Involvement of lymph node regions on both side of diaphragm  |
| <b>IV</b>                  | Involvement of extranodal site(s) beyond that designated E   |
| <b>For all stages</b>      |  |
| <b>A</b>                   | No symptoms  |
| <b>B</b>                   | Fever (>38°C), drenching sweats, weight loss (10% body weight over 6 months)                           |
| <b>For Stages I to III</b> |  |
| <b>E</b>                   | Involvement of a single, extranodal site contiguous or proximal to known nodal site                    |

#### 1.5 - **Prognosis**

Prognostic information for most NHL is not accurately provided by the Ann Arbour system and it is of limited value for treatment decisions <sup>(24)</sup>.

In 1993, the International Prognostic Index (IPI) <sup>(25)</sup> was developed as a primary clinical tool to predict outcome in patients with aggressive NHL <sup>(26)</sup>. This classification is based on some negative prognostic factors at the time of diagnosis (Table 3). It includes age over 60 years, Eastern Cooperative Oncology Group (ECOG) poor performance status

(Table 4), Ann Arbor Stage I and II versus Stage III or IV, elevated serum lactate dehydrogenase (LDH) and involvement of two or more extranodal sites as high-risk factors (24, 25, 26).

**Table 3 – International Prognostic Index. Adapted from (1,24).**

| Prognostic Factors  | Risk Category Factors  |
|---|--|
| <ul style="list-style-type: none"> <li>• Age &gt; 60 years</li> <li>• Performance status &gt; 2</li> <li>• Lactate dehydrogenase 1x normal</li> <li>• Extranodal sites &gt; 2</li> <li>• Stage III or IV</li> </ul> | <ul style="list-style-type: none"> <li>• Low (0 or 1)</li> <li>• Low-intermediate (2)</li> <li>• High-intermediate (3)</li> <li>• High (4 or 5)</li> </ul> |

**Table 4 – Eastern Cooperative Oncology Group Performance Status. Adapted from (24, 28).**

| Grade | Description  |
|-------|--|
| 0     | Fully active, able to carry on all predisease performance without restriction  |
| 1     | Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature (eg, office work) |
| 2     | Ambulatory and capable of self-care but unable to carry out any work activities. Up and about more than 50% of waking hours            |
| 3     | Capable of only limited self-care, confined to bed or chair more than 50% of waking hours  |
| 4     | Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.  |
| 5     | Dead   |

The IPI stratified DLBCL into four discrete outcome groups with a 5-year overall survival (OS) ranging from 26 to 73% (25,26, 27). Further studies demonstrated that a better risk discrimination was achieved with a re-categorized IPI (R-IPI) for rituximab-based immunochemotherapy (26). Thus, Sehn *et al* stratified IPI into three categories with significantly different outcome:

- “Very good” (score 0) – patients with zero risk factors, with more than 90% chance of long-term progression-free survival
- “Good” (1 or 2) – patients with one or two risk factors, with approximately 80% chance of long-term progression-free survival
- “Poor” (3-5) – patients with three, four or five risk factors, with a 50% chance of long-term progression-free survival.



Risk group with less than 50% likelihood of long-term progression-free survival is not identified by neither IPI or R-IPI <sup>(26)</sup>.

Many studies have subsequently focused on recognizing novel molecular and genetic markers to define more precise prognostic factors. Although several molecular prognostic markers have been identified, they have still not been validated <sup>(29,30,31)</sup>. Thus, IPI and R-IPI remain the most reliable tools for predicting outcome in patients with DLBCL <sup>(26)</sup>, and it is recommended that evaluation of a new patient should involve both systems: Ann Arbor Staging and International Prognostic Index.

## **1.6 – Therapy**

Although DLBCL are very aggressive, they are potentially curable with multimodal chemotherapy <sup>(1)</sup>. The cyclophosphamide, hydroxydaunorubicin, oncovin and prednisone (CHOP) chemotherapy regimen has been considered the standard treatment for several decades <sup>(32)</sup>. In 1994, the results from Czuczman *et al* encouraged the use of rituximab with CHOP (rituximab, cyclophosphamide, hydroxydaunorubicin, oncovin, prednisone - R-CHOP) for indolent lymphoma <sup>(34)</sup>. Subsequently, improved failure-free survival (FFS) and OS with R-CHOP (administered as rituximab on day 1 of each of eight CHOP cycles) compared with CHOP, especially in older patients, was disclosed <sup>(32, 35, 36)</sup>. Thus, R-CHOP is currently the standard of care in treatment of older patients with DLBCL. Toxicity data and efficacy associated with the number of chemotherapy cycles and the number of rituximab infusions must, however, be considered <sup>(7,32,33)</sup>. Nevertheless, about 35% to 50% of patients with advanced-stage disease are not cured using R-CHOP <sup>(8)</sup>. Therefore, it is important to understand and make efforts to identify biologic subgroups among DLBCL and develop more appropriate treatments to address DLBCL heterogeneity <sup>(32)</sup>.

## 2 – EPIGENETICS

“Epigenetics”, a term firstly used by Conrad Waddington <sup>(37,87)</sup>, consist on the study of heritable changes in gene expression, involving active and inactive genes, without change in the DNA sequence. There is a change in phenotype without a change in genotype <sup>(39, 40, 41)</sup>. Epigenetic change is a regular and natural occurrence but can also be influenced by several factors including age and the environment/lifestyle <sup>(40)</sup>. Epigenetic change can induce damaging effects, helping the cell to adapt or promoting pathological behaviour, that can result in cancer formation. It may occur at distinct stages of tumorigenesis and contribute to the development or progression of cancer. There is variable heterogeneity in epigenetic alterations that may give similar phenotypes. Thus, the varied clinical behaviour of cancers can range from indolent, slow-growing to aggressive, fast-growing tumors <sup>(41)</sup>.

The epigenetic mechanism embrace three main systems: DNA methylation, histone modification and non-coding RNA (ncRNA)-associated gene silencing, that are currently considered to initiate and sustain epigenetic change <sup>(40)</sup>.

### 2.1 – DNA Methylation

DNA methylation is one of the most well studied and characterized epigenetic modifications, which consists on the addition of a methyl group to carbon 5 of the cytosine residue within the nucleotide cytosine-phosphatidyl-guanine (CpG) <sup>(43, 44)</sup> (Figure 6). CpG methylation of DNA is found at sites with a high percentage of CpGs (so-called CpG islands), leading to transcriptional repression <sup>(40)</sup>.

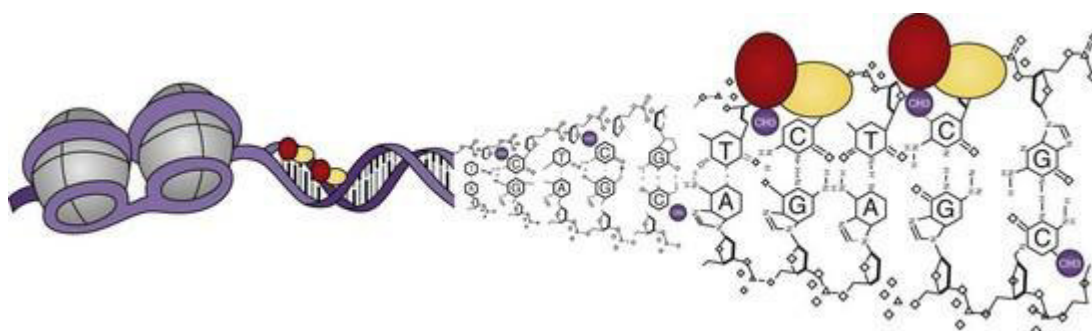


Figure 6 – DNA methylation. DNA gets methylated at the pyrimidine ring of the cytosine. The methyl mark leads to changes in the chromatin structure and recruits effector molecules. (A-adenosine; C-cytosine; G-guanine; T-thymine). Adapted from <sup>(40)</sup>.

*De novo* methylation marks are established by DNA methyltransferase (DNMT) 3a and 3b, through embryonic development. The formation of heterochromatin and gene

silencing depends on DNA methylation <sup>(40, 44)</sup>. In almost all forms of cancer aberrant hypermethylation and silencing of tumor suppressor genes has been found. Both hypometylation and hypomethylation of CpG islands located at gene promoters may affect the expression of protein coding genes and of non-coding RNA's, eventually resulting in tumorigenesis <sup>(44-47)</sup>.

## 2.2 – Histone modification

The second major mechanism in epigenetic gene regulation is modification of histone tails by chromatin modifying enzymes, that has significant impact on intra and inter-nucleosomal interactions <sup>(44,48,49,50)</sup>. DNA is packaged around an octamer comprising homodimers of four different histone proteins: H2A, H2B, H3 and H4. The nucleosome is stabilized in place by H1. Every histone protein has an N-terminal tail, which is the main target of a diversity of histone modifications <sup>(40)</sup> (Figure 7).

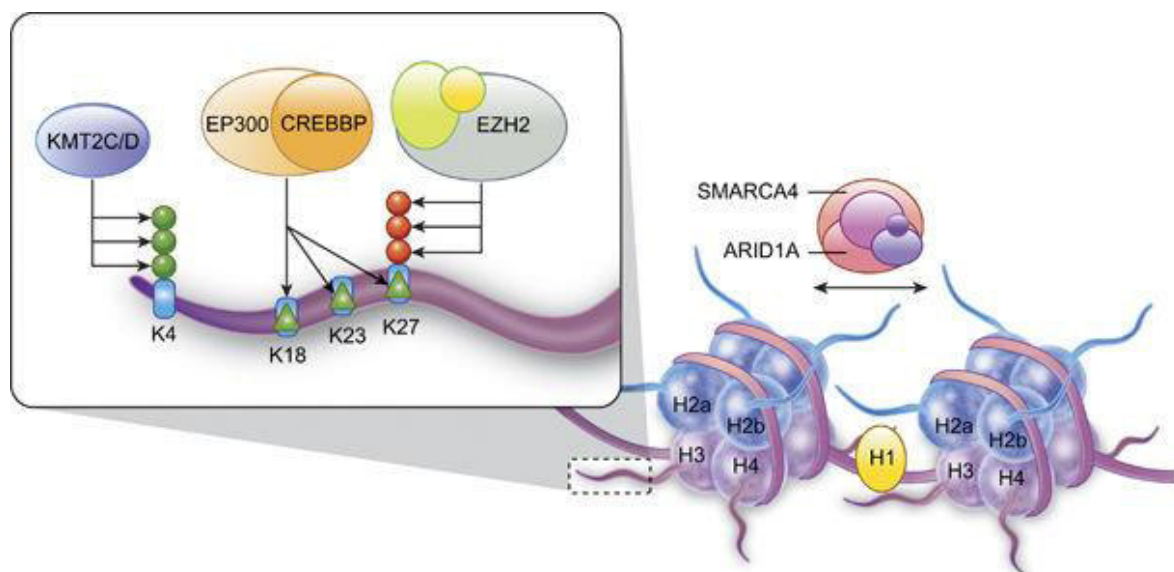


Figure 7 -Function of chromatin modifying and organizing genes. DNA is wrapped around histone octamers to form a nucleosome. Adapted from <sup>(50)</sup>.

Modifications of histones can make changes into two ways: modifications can be recognized by “readers” that can recruit additional factors like other chromatin-modifying enzymes, and acetylation and phosphorylation may act on chromatin structure by reducing

the positive charge of histones and alter their association with negatively charged DNA <sup>(51-55)</sup>.

### 2.2.1 – Histone lysine methylation

Histone lysine methylation may occur at residues 4, 9, 27, 36 and 79 of Histone 3 and residue 20 of Histone 4 <sup>(56)</sup>. Three degrees of methylation are known: mono-methylation (me1), di-methylation (me2) or trimethylation (me3) that may be associated with active euchromatin or inactive heterochromatin states <sup>(40,57)</sup>. H3K4 methylation is frequently associated with active transcription and, thus, H3K4me3 is usually located around promotor regions whereas H3K4me1 is located around enhancer regions. In contrast to H3K4me3, trimethylation of lysine 27 on histone H3 (H3K27me3) is associated with transcriptional repression. Histone methylation does not have a direct effect on chromatin structure <sup>(57-59)</sup>.

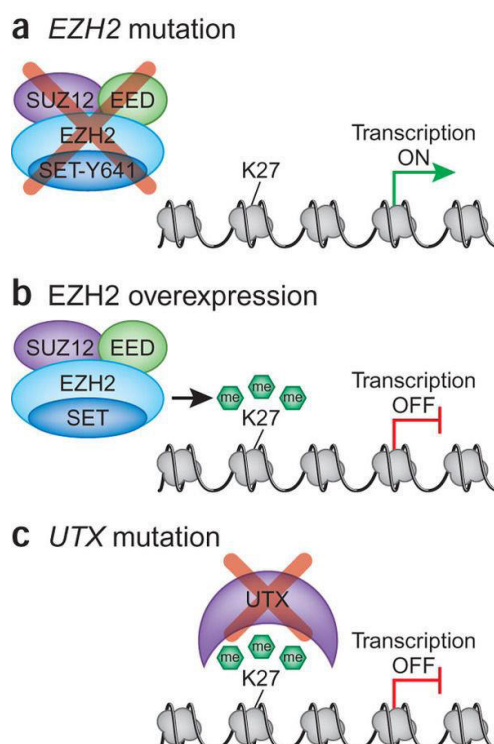
Histone lysine methylation is also a dynamic process that encodes a diversity of chromatin states <sup>(40)</sup>.

#### ➤ H3K4 methylation

One of four members in the mixed lineage leukemia (MLL) family proteins - KMT2D (MLL2) – is involved in H3K4 methylation. KMT2D somatic mutations are found in most follicular lymphomas (FL) but is less common in GCB-like DLBCL. The polycomb repressor complex 2 is unable to methylate H3K27me3 if H3K4 is trimethylated in the same histone tail. The H3K4me3 is recognized by reader proteins such as the product of ING1 gene, which is deleted in 1/3 of GCB-like DLBCL. Until now, there is no evidence of the effect of KMT2D mutation on H3K4 methylation or a suggestion of the mechanism by which these mutations promote lymphomagenesis <sup>(40,51,60)</sup>.

#### ➤ H3K27 methylation

Enhancer of zeste homolog 2 (EZH2) is the enzymatic subunit of an epigenetic gene-silencing complex named polycomb repressive complex 2 (PRC2). It is a SET domain histone methyltransferase that catalyses histone 3 lysine 27(H3K27) methylation, leading to a transcriptional repression of the target gene <sup>(50, 57, 61-67)</sup>.



**Figure 8 – Deregulation of H3K27 methylation in cancer.** A – EZH2 trimethylates H3K27 to inhibit gene expression; loss of EZH2 in cancer may lead to derepression of genes that promote cell growth. B – EZH2 overexpression silences additional targets. C – UTX removes H3K27me3 marks, loss of UTX increases H3K27me3 and silences tumor suppressors. Adapted from <sup>(57)</sup>.

Components of PRC2 are highly expressed in germinal centers. The first chromatin-modifying gene alteration described in DLBCL was *EZH2* mutation. An increase or decrease of H3K27 methylation activity may lead to malignancy, depending on the cell of origin of the tumor <sup>(57, 67-69)</sup>. The H3K27me3 mark is self-sustaining, as DNA replicates, H3K27 methylated histones recruits PRC2 and PRC1 complexes to nucleosomes of the nascent DNA strand to continue gene silencing <sup>(70)</sup>. Heterozygous *EZH2* mutations suggest that cancer cells were haploinsufficient for the enzymatic activity. It can result in deficient H3K27 methylation and widespread derepression of gene expression. Overexpression of EZH2 may cause silencing of growth-suppressive genes <sup>(71)</sup>. Some studies propose that increased or decreased H3K27 methylation activity may lead to malignancy, depending on the cell of origin <sup>(72,73, 74)</sup>.

### **2.3 – Epigenetics of diffuse large B-cell lymphoma**

Complex diseases require clarifications about genetic and environmental influences in the causative factors and prediction parameters. Epigenetic code alterations have been found in diverse pathological conditions <sup>(55,75)</sup>, including cancer <sup>(76)</sup>. In DLBCL, it was demonstrated that EZH2 is important for G2/S transition and represses cell cycle-related tumor suppressor genes, through trimethylation of H3K27, contributing disease progression <sup>(77)</sup>. Mutations of EZH3 were identified in 22% of GCB DLBCL <sup>(55, 58, 64, 68)</sup>. The most frequent EZH2 mutations are found in the SET domain of EZH2 affecting tyrosine 641 (Y641), alanine 677 (A677) and alanine 687 (A687). EZH2 gain-of-function mutations favours increased H3K27me3 levels and loss-of-function mutations were identified in H3K4 trimethylase MLL2 in 32% of DLBCL <sup>(80,81)</sup>. Whereas H3K27 trimethylation due to EZH2 activity has been associated with gene repression, methylation of H3K4 was associated with gene expression, with MLL and EZH2 displaying opposite functions <sup>(82)</sup>. Indeed, elevated levels of H3K27me3 in approximately one third of the DLBCL cases has been reported, suggesting that it may be involved in a subgroup of DLBCL. Moreover, an elevated level of H3K27me3 was associated with poor overall survival within non-GCB DLBCL patients <sup>(83)</sup>.

## **AIMS OF THE STUDY**





As previously stated, altered activity of MLL and EZH2 has been reported in DLBC lymphoma, affecting the levels of the respective marks, H3K4me3 and H3K27me3, eventually causing altered expression of several critical genes. Thus, we hypothesized that quantitative evaluation of H3K4me3 and H3K27me3 might constitute biomarkers of DLBC progression, providing novel prognostic parameters for risk stratification of patients with DLBCL.

Hence, the specific aims of this Dissertation are:

- To evaluate quantitatively the immunohistochemical expression of histone-marks H3K4me3 and H3K27me3 in diffuse large B-cell lymphoma;
- To correlate the immunohistochemical expression with standard histopathological and clinical parameters, as well as disease-specific and overall survival;
- To verify their potential value as prognostic biomarkers for patients with diffuse large B-cell lymphoma.



## **MATERIALS AND METHODS**



## 1 - PATIENTS AND SAMPLES

For the purposes of this retrospective study, patients diagnosed (Department of Pathology) and treated (Department of Onco-Hematology) for diffuse large B-cell lymphoma at Portuguese Oncology Institute of Porto, Portugal between 2008 and 2013 were identified. Then, all cases with available representative archived tissue were enrolled, corresponding to 155 patients.

Pertinent clinical data were collected from the clinical charts by an experienced Hematologist (Dr. Sérgio Chacim) from the Department of Onco-Hematology.

This study, the use of samples and access to clinical data were approved by the institutional review board (Comissão de Ética para a Saúde) of Portuguese Oncology Institute of Porto, Portugal.

## 2 – IMMUNOHISTOCHEMISTRY

Three  $\mu\text{m}$  thick paraffin sections were obtained from representative formalin-fixed paraffin embedded tissue from each case, and placed in positively charged slides (Histobond®, Marienfeld, Germany) for immunohistochemistry assays.

For deparaffinization, slides were placed in an oven at 60°C for 1 hour and 30 minutes, and then immersed in xylene (Sigma-Aldrich®, St. Louis, MO, USA). Subsequently, sections were hydrated in decreasing series of ethanol solutions (Merck, Darmstadt, Germany).

### 2.1 - Epitope retrieval and endogenous peroxidase neutralizing

For antigens retrieval, slides were placed in sodium citrate buffer (1:100; pH6; Vector® Antigen Unmasking Solution; H-3300; Burlingame; CA 94010 U.S.A) in a Bosch microwave oven at 700W (2 cycles of 10 minutes each).

Endogenous peroxidase activity was neutralized with 0.6% hydrogen peroxide for 20 minutes (Merck).

Protein detection was performed using the Novolink™ Max Polymer Detection System (Leica Biosystems, Nussloch, Germany), according to a standard protocol (Annex 1). Depending of the antibody used, the incubation period varied, as depicted in Table 5.

**Table 5 – Antibodies and Conditions**

| Antibodies | Company                   | Clone                                     | Positive Control         | Dilution | Incubation                         |
|------------|---------------------------|---|--------------------------|----------|------------------------------------|
| H3H4me3    | Abcam                     | Rabbit polyclonal-ChIP Grade ab8580       | Hepatocellular carcinoma | 1:1000   | 1h<br>(Humid chamber)              |
| H3K27me3   | Cell Signaling Technology | Rabbit monoclonal antibody (mAb) - C36B11 | Colorectal cancer        | 1:1500   | Overnight<br>(4°C – Humid chamber) |

Successive washing steps were performed with tris buffered saline with Tween® 20 (TBS-T) (Sigma-Aldrich®).

Antigen-antibody binding reaction was revealed by means of incubation with a 0.05% (m/v) 3,3'- diaminobenzidine (DAB) solution (Sigma-Aldrich®) in phosphate-buffered saline (PBS) (Biochrom Ltd., Cambridge, United Kingdom) previously activated with a 0,1% hydrogen peroxide solution, for 7 minutes, in the dark.

Counterstaining of the slides was performed with hematoxylin (Merck) for 30 seconds and then slides were washed for 1 minute in a tap water.

Finally, slides were dehydrated in an increasing series of ethanol solutions, diaphanized in xylene and mounted.

Negative control consisted on the omission of the primary antibody. Appropriate positive controls (see Table 5) were used in each batch.

Positive control slides were evaluated in an optical microscope by an experienced Pathologist.

### 3 - NUCLEAR IMMUNOSTAINING QUANTIFICATION

The nuclear immunostaining quantification was done by a digital image analysis system (GenASIs, Version 7.2.7.31510, ASI, “Applied Spectral Imaging”, 2013) (Figure 9).



Figure 9 - Equipment support for the digital image analysis system GenASIs™.

The slides were scanned at 200x magnification and high-resolution digital images were obtained. Manual selection of 5 to 8 areas of tumour cells were captured to reach at least 5000 cells fit for evaluation (Figure 10).

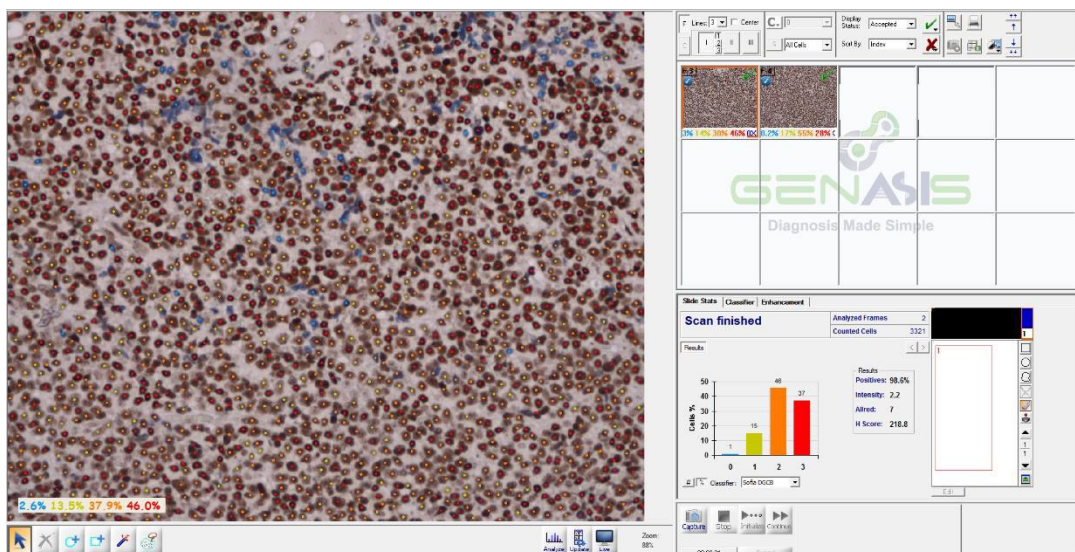


Figure 10 – Example of capture digital images on GenASIs™ software.

Subsequently, positive nuclei were automatically detected and a nuclear classification into 4 levels of intensity (0-3) was generated, each one identified with a different color (Figure 11):

- 0 (negatively stained nuclei) – blue;
- 1 (low intensity stained nuclei) – yellow;
- 2 (intermediate intensity stained nuclei)– orange;
- 3 (higher intensity stained nuclei) – red.

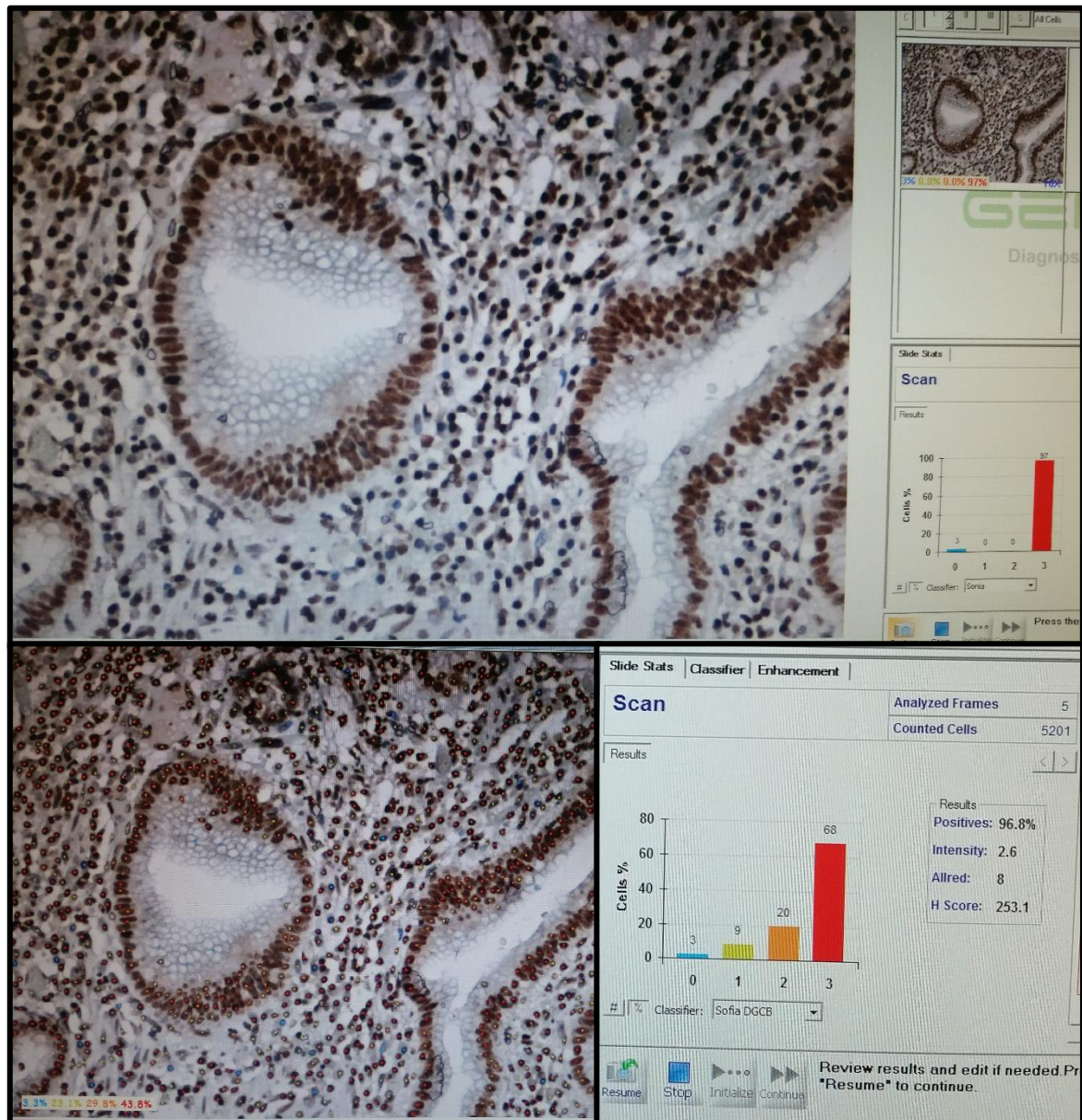


Figure 11 – Example of nuclear classification into 4 levels of intensity on GenASIs™ software.



The percentage of immunostained nuclei and the H-score were determined for each case and used for subsequent analyses.

#### **4 - STATISTICAL ANALYSIS**

For statistical analysis, patients were divided into two Ann Arbour Stage groups (stage I/II, stage III/IV) and into three IPI scores (0/1-2/3-5). Cutoffs for H3K4me3 and H3K27me3 nuclear immunostaining (high vs. low) were set at 25<sup>th</sup> and 75<sup>th</sup> percentile (P25 and P75). Comparison between % Positivity, Hscore, clinical and pathological parameters was performed using non-parametric tests (Mann-Whitney or Kruskal-Wallis, for IPI). Survival curves were created using Kaplan-Meier non-parametric estimator. Survival between groups was compared using Log-rank test. Statistical significance was considered when p-value was inferior to 0.05. All statistical analyses were performed using R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria).



## **RESULTS**



## 1 – CLINICAL AND PATHOLOGICAL CHARACTERIZATION OF THE PATIENTS

For the purposes of this study, 155 patients diagnosed with DLBCL and treated at the Portuguese Oncology Institute of Porto, from 2008 to 2013 were included. The clinical and pathological characteristics of this cohort are shown in Table 6. In this cohort, 43.6% of patients were male, and the median age at diagnosis was 66 years. Recurrent/transformed disease was experienced by 40 % of the patients.

**Table 6 - Clinical and pathological characteristics of the patients**

|                         |                         | <b>N</b> | <b>%</b> |
|-------------------------|-------------------------|----------|----------|
| <b>Gender</b>           | Female                  | 87       | 56,1%    |
|                         | Male                    | 68       | 43,9%    |
| <b>Age at Diagnosis</b> | ≤ 70 years              | 99       | 63,9%    |
|                         | > 70 years              | 56       | 36,1%    |
| <b>Ann Arbour Stage</b> | I/II                    | 79       | 50,6%*   |
|                         | III/IV                  | 72       | 46,2%*   |
| <b>IPI</b>              | Low risk (0)            | 21       | 13,5%**  |
|                         | Intermediate risk (1,2) | 66       | 42,6%**  |
|                         | High risk (3-5)         | 51       | 32,9%**  |

\*3,2% of patients has unknown Ann Arbour Stage information.

\*\* 11% of patients has unknown IPI information.

## 2 –SURVIVAL ANALYSIS

The follow-up time was determined at date of the last consultation and the median was 55.4 months. The overall survival was estimated as 76.7% at 2 years and 68.1% at 5 years. The disease-specific survival was estimated as 79.1% at 2 years and 74.9% at 5 years.

For survival analysis, we considered the association between the disease-specific survival and the Ann Arbour stage, IPI score, and age at the time of diagnosis (≤ or > 60 years and ≤ or > 70 years). In our dataset, individualized Ann Arbour stage was not associated with prognosis ( $p=0.088$ ) (Figure 12), whereas grouped Ann Arbour stage (stages I/II vs. III/IV) was associated with prognosis ( $p=0.015$ ).

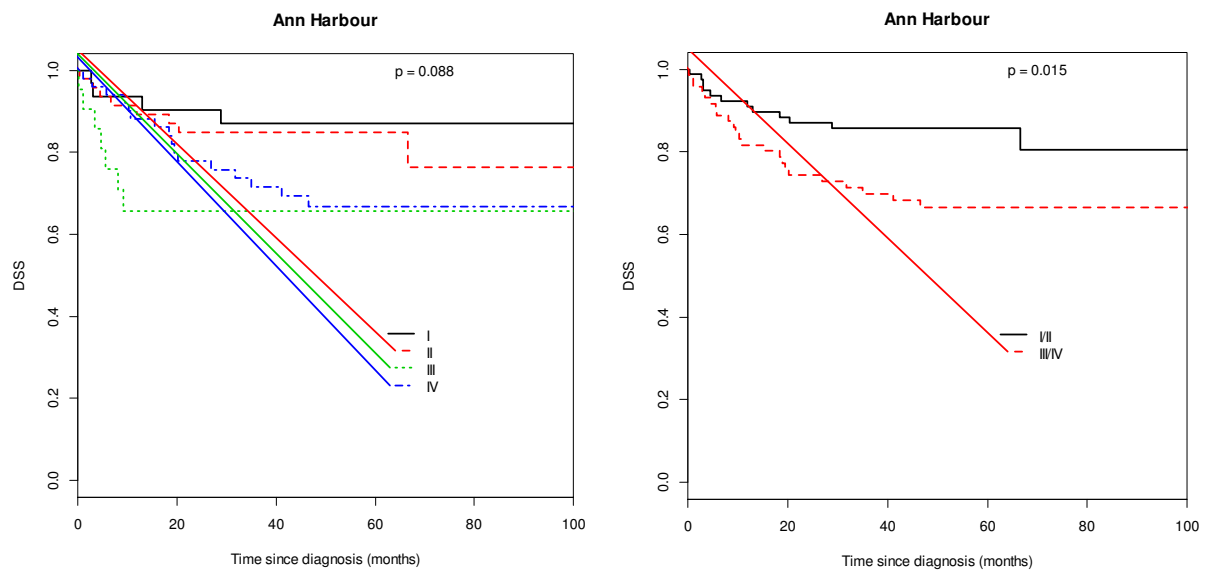


Figure 12 - Analysis of disease-specific survival according to individualized or grouped Ann Arbour stage.

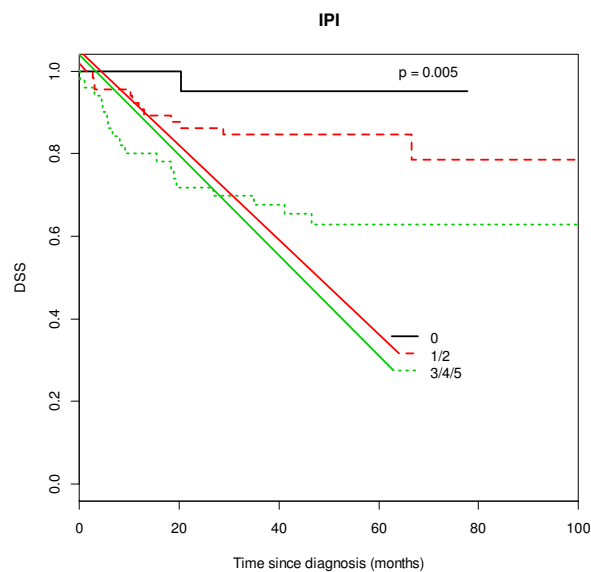


Figure 13 - Analysis of disease-specific survival according to grouped IPI score.

IPI score ( $p=0.005$ ) and age at the time of diagnosis ( $\leq$  or  $> 60$  years and  $\leq$  or  $> 70$  years) were significantly associated with prognosis ( $p=0.006$  and  $p=0.005$ , respectively) (Figures 13 and 14). Indeed, lower IPI score and age at diagnosis were associated with a better outcome.

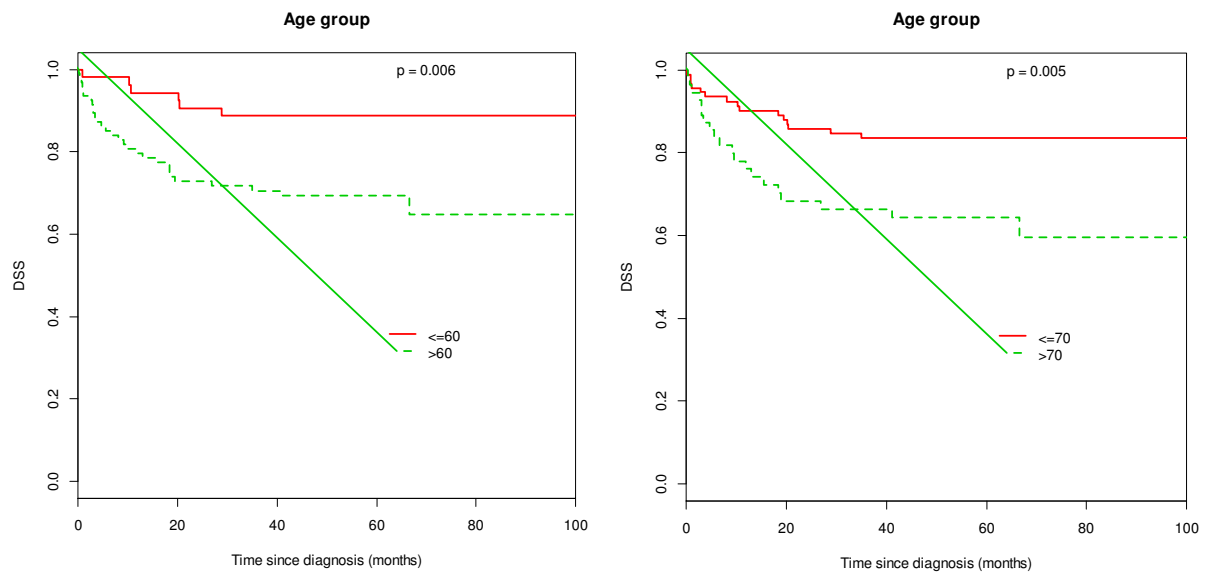


Figure 14 - Analysis of disease-specific survival according to age ( $\leq$  or  $>$  60 years and  $\leq$  or  $>$  70 years).

No difference in prognosis was observed concerning gender ( $p=0.154$ ) (Figure 15).

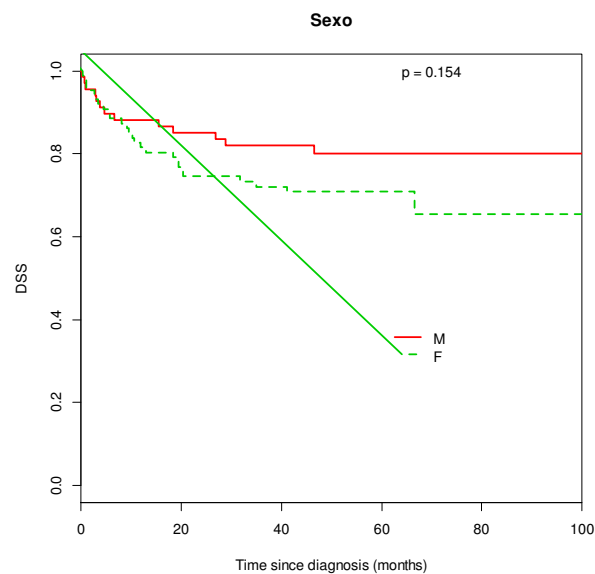


Figure 15 - Analysis of disease-specific survival according to gender.

### 3 – IMMUNOHISTOCHEMICAL PROFILE

The percentage of immunostained nuclei and the H-score were determined for each case and used to evaluate the immunoexpression profile.

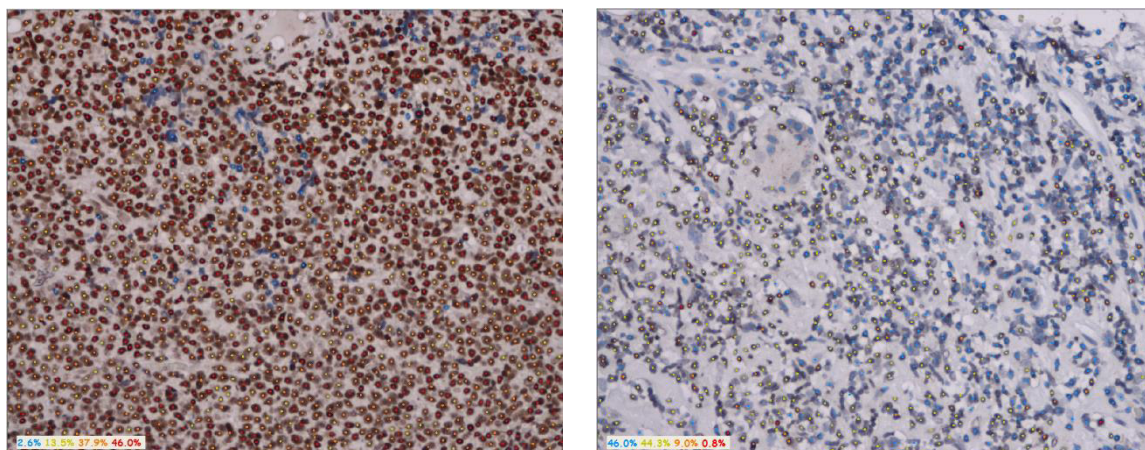
- H3K4me3

For H3K4me3 epigenetic mark, Hscore varied between 4.1 and 276.2, with a median value of 157.1 (Figure 16). In statistical analysis of the distribution of Hscore of H3K4me3 according to gender, age, Ann Arbour stage, transformation and IPI there were no statistically significant associations. We used Mann Whitney's test for all the variables except for IPI score, in which Kruskal Wallis' test was used (Table 7).

**Table 7 - Comparison between Hscore, clinical and pathological parameters.**

|                               | P     |
|-------------------------------|-------|
| Gender                        | 0.216 |
| Age ( $\leq$ or $>$ 70 years) | 0.118 |
| Ann Arbour Stage              | 0.148 |
| Transformation                | 0.536 |
| IPI                           | 0.472 |

The immunoexpression of H3K4me3 was not associated with disease-specific survival using either % Positivity and Hscore median ( $p=0.643$  and  $p=0.917$ , respectively) or the 25<sup>th</sup> and 75<sup>th</sup> percentile for Hscore ( $p=0.978$  and  $p=0.518$ , respectively) (Figures 18, 19 and 20).



**Figure 16 - Positive and negative nuclear immunostaining of H3K4me3.**



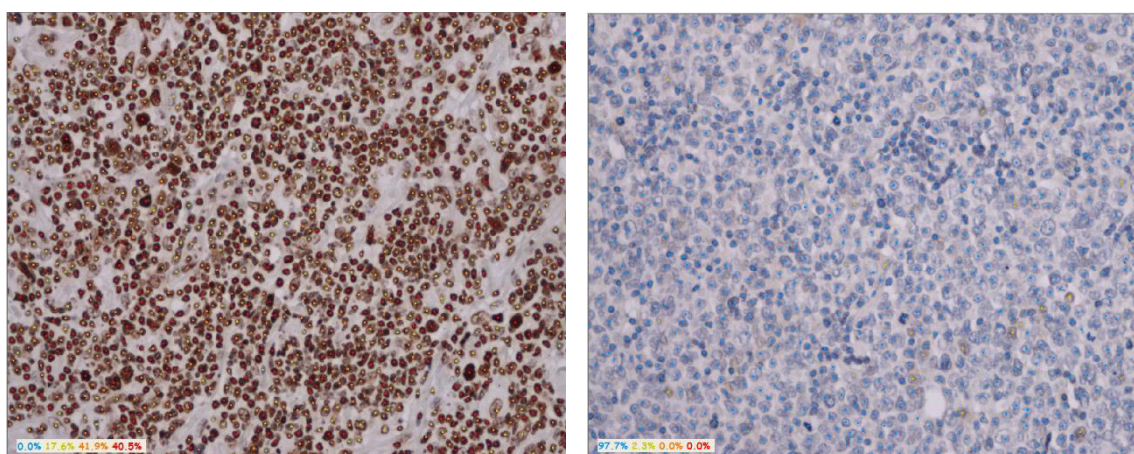
- H3K27me3

Concerning H3K27me3 epigenetic mark, Hscore varied between 38.6 and 247, with a median of 117.2 (Figure 17). In statistical analysis of the distribution of Hscore of H3K4me3 according to gender, age, Ann Arbour stage and transformation there were no statistically significant results except for IPI score ( $p = 0.041$ ). We used Mann Whitney's test for all the variables except for IPI score, in which Kruskal Wallis' test was used (Table 8).

**Table 8 - Comparison between Hscore, clinical and pathological parameters.**

|                               | P            |
|-------------------------------|--------------|
| Gender                        | 0.634        |
| Age ( $\leq$ or $>$ 70 years) | 0.530        |
| Ann Arbour Stage              | 0.094        |
| Transformation                | 0.422        |
| IPI                           | <b>0.041</b> |

The immunoexpression of H3K27me3 was not associated with disease-specific survival using either the % Positivity and Hscore median ( $p=0.979$  and  $p=0.959$ , respectively) or the 25<sup>th</sup> and 75<sup>th</sup> percentile ( $p=0.291$  and  $p=0.683$ ), respectively) (Figures 18, 19 and 20).



**Figure 17 - Positive and negative nuclear immunostaining of H3K27me3.**

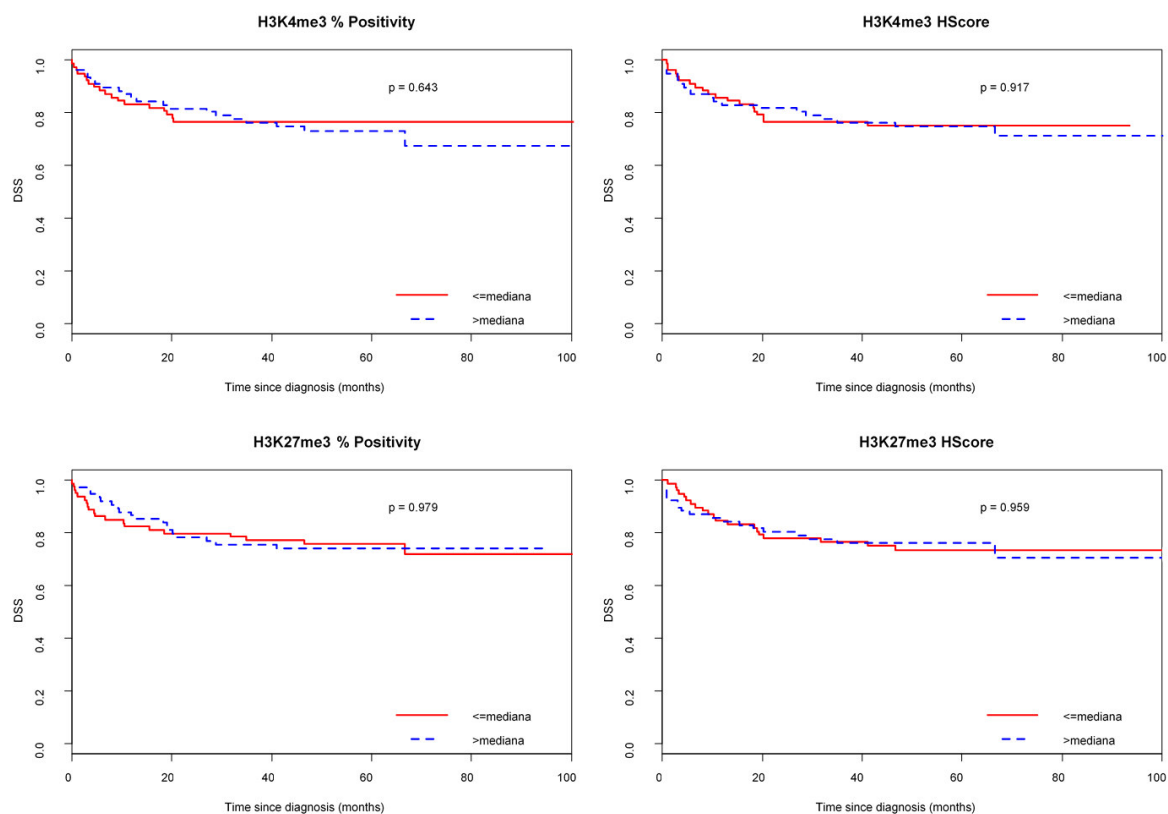


Figure 18 - Analysis of disease-specific survival by % Positivity, in the left panel and Hscore, on the right panel. The comparison is made using the median.

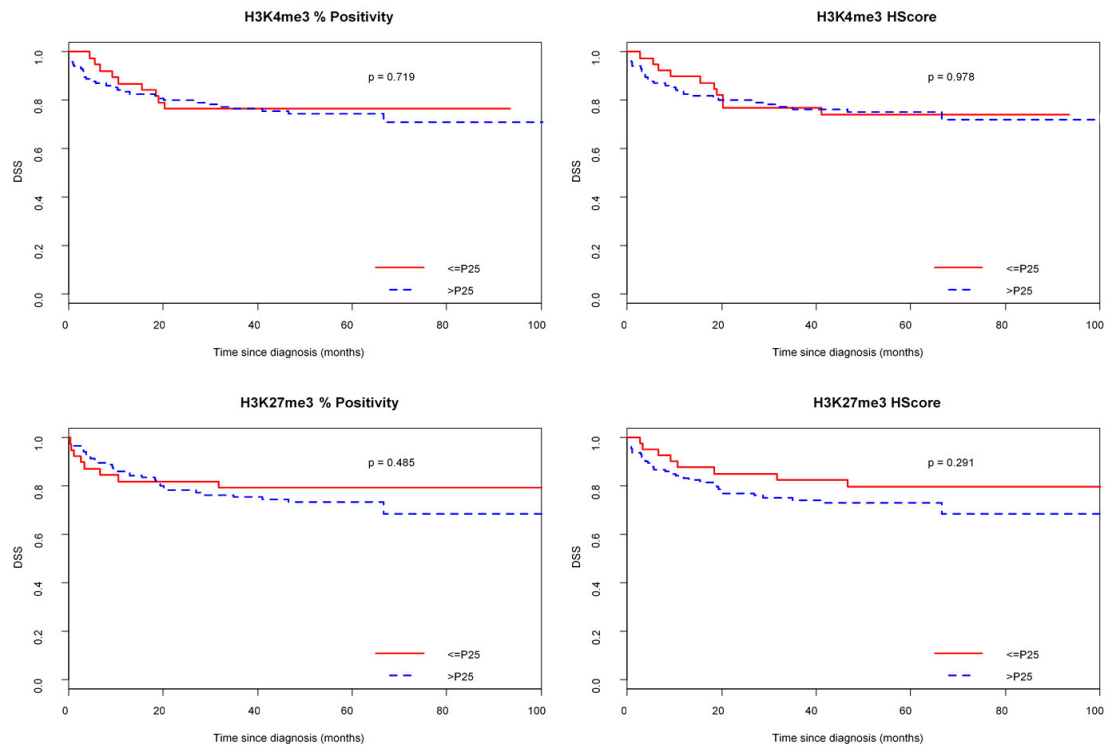


Figure 19 - Analysis of disease-specific survival by % Positivity, in the left panel and Hscore, on the right panel. The comparison is made using the 25<sup>th</sup> percentile.

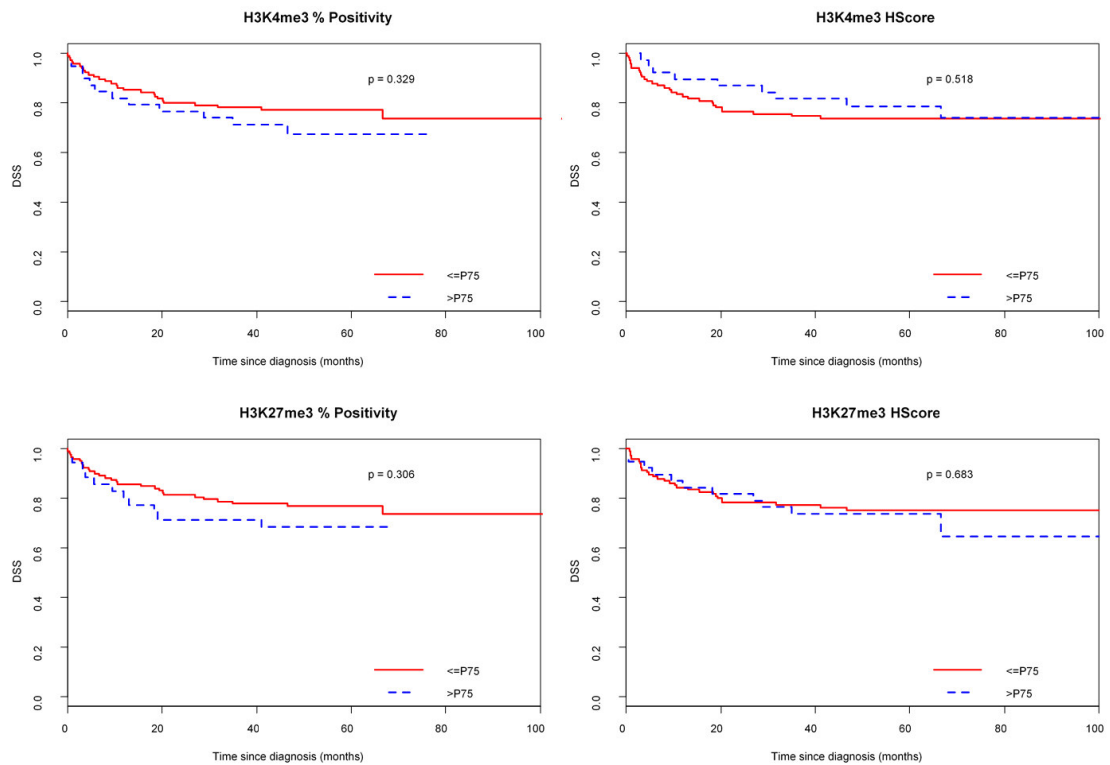


Figure 20 - Analysis of disease-specific survival by % Positivity, in the left panel and Hscore, on the right panel. The comparison is made using the 75<sup>th</sup> percentile.



## **DISCUSSION**



Diffuse large B-cell lymphomas are a heterogeneous group of lymphoid neoplasms that have a wide range of clinical presentations, immunologic characteristics, genetical features and clinical outcomes in patients that experience standard therapy<sup>(8)</sup>. It is important to emphasize the diversity of DLBCL as it must be taken into consideration when investigating new therapies<sup>(9, 27, 33)</sup>. Currently, prognosis of patients with DLBCL is estimated using the clinical parameters of the International Prognostic Index<sup>(27)</sup>. The predictive ability of IPI is, however, limited and estimating the risk that a DLBCL poses to an individual patient remains a challenging task. Thus, improved means of prognostication are critical to allow for individualized risk-adapted therapy. Likewise, development of predictive biomarkers will be essential to select appropriate patients for novel target approaches with the goal of achieving a personalized cancer care approach<sup>(33,47)</sup>. Taking in consideration these principles and considering that altered patterns of histone modifications associated with EZH2 and MLL2 mutations occur in DLBCL<sup>(55, 58, 64, 68)</sup>, we hypothesized that a quantitative evaluation of histone marks, specifically H3K4me3 and H3K27m3, might provide candidate prognostic biomarkers which might be used as ancillary tools for risk-stratification of DLBCL patients.

This study was based in a consecutive series of 155 patients diagnosed with DLBCL and treated in IPO Porto between 2008 and 2013. The median age at diagnosis, 66 years, and the percentage of patients more than 70 years old (36,1%) are in accordance with published literature<sup>(11)</sup>. However, the observed M/F ratio was 1/1.3, which is an inverse proportion compared to most published studies<sup>(1,2,14)</sup>. There is no clear explanation for this finding, which might bias subsequent analyses. Nevertheless, gender is not considered a prognostic factor in DLBCL and, thus, we are confident that the bias, if it exists, it is likely to be negligible. Indeed, more relevant parameters including the distribution of IPI score (13.5%, 42.6% and 32.9%) and the proportion of patients with transformation/recurrence (40%) are similar to those of previous studies<sup>(27,1,5)</sup>. Sample size, however, might account for some results, such as the lack of association of individualized Ann Arbor stage (i.e., stages I vs. II vs. III vs. IV), which was, however, disclosed when stages I/II and III/IV were lumped together. Moreover, and as expected, IPI score and age at the time of diagnosis (both  $\leq$  or  $>$  60 years and  $\leq$  or  $>$  70 years) were significantly associated with prognosis, providing indirect validation of this patient cohort.

The main goal of this study was to investigate the potential of quantitative H3K4me3 and H3K27me3 immunoexpression as prognostic biomarker in DLBCL, which, to the best of our knowledge, has not been attempted before. The rationale for this approach was that the enzymes that catalyse H3K4me3 and H3K27me3, specifically MLL2 and EZH2, display altered activity in a proportion of DLCL cases. Whereas the EZH2 activating mutations, which were found in up to 22% of DLCL<sup>(55, 58, 64, 68)</sup>, are deemed to increase H3K27me3, MLL2 inactivating mutations, occurring in 32% of DLBCL<sup>(80,81)</sup>, are expected to decrease H3K4me3. Di- and trimethylation of H3K4 is linked to transcriptional activation and high levels of H3K4me3 trimethylation are associated with the promoters of actively transcribed genes<sup>(57-59)</sup>. H3K27me3, on the other hand, is an epigenetic mark associated with transcriptional silencing by promoting a compact chromatin structure<sup>(57-59)</sup>. Thus, both alterations might lead to inappropriate silencing of genes that are critical for the control of cell growth and proliferation. We expected that more aggressive DLCL would associate with lower and/or higher H3K4me3 and H3K27me3 Hscore/percentage of immunostained cells, respectively. However, only a significant association between IPI and H3K27me3 Hscore was found. Moreover, survival analysis did not disclose any association between quantitative H3K4me3 and H3K27me3 immunoexpression and patients' outcome, although several cutoff values were tested (P25, median, P75). A previous study has shown an association between increased H3K27me3 and poor overall survival in DLBCL<sup>(83)</sup> and in other tumor models increased EZH2 expression has been associated with worse prognosis and an increase of invasion and tumour progression capacities<sup>(41)</sup>. Nevertheless, it is noteworthy that the association observed between increased H3K27me3 expression and poor overall survival was restricted to non-GCB DLBCL patients<sup>(83)</sup>. Our cohort includes both GCB and non-GCB DLBCL, which we did not discriminate owing to the lack of sufficient immunophenotypical information. Thus, a direct comparison between the two studies cannot be made. Moreover, our negative results might also derive from an insufficient sample size, not having statistical power to disclose the previously reported associations. It should also be recalled that the immunohistochemical assay used in our study provides a global estimate of H3K4me3 and H3K27me3 expression, which might not reflect important gene-specific silencing events, which we are not able to identify with this methodology. However, the selection of an immunohistochemical assay was based on the widespread availability of this technique in most Pathology labs, eventually facilitating translation into routine clinical practice.

Several limitations of this study should be acknowledged. Firstly, and as previously stated, sample size may not be sufficient to ascertain a significant association between



H3K4me3 and H3K27me3 immunoexpression and prognosis. In some cases, information about prognosis factors (Ann Arbour Stage and IPI) was missing and this may negatively influence statistical analyses. Furthermore, the absence of discrimination of DLCL according to the cell of origin, i.e., using the immunohistochemical assays for CD10, BCL6 and MUM1 as surrogate markers to identify GCB and non-GCB subtypes, precluded a more precise and detailed evaluation of the potential prognostic value of the epigenetic marks tested. Nevertheless, an important and innovative feature of this study must also be emphasized: the use of quantification of immunoexpression by means of a digital image assisted system (GenASIs™) which is able to provide more reliable and reproducible results. This type of approach is seldom used in routine pathological practice but its use should be encouraged to increase the reliability of several immunohistochemical parameters that are currently used to assist in therapeutic decision.



## **CONCLUSIONS AND**

## **FUTURE PERSPECTIVES**



Considering our results, we may conclude that in our patient cohort, that globally may be considered representative of DLCL patients, quantitative H3K4me3 and H3K27me3 immunoexpression were not disclosed as candidate prognostic biomarkers.

The next steps of this work have already been defined. First, the cohort will be increased with the addition of more cases, to allow for a more powerful statistical analysis. Secondly, discrimination of GCB and non-GCB subtypes will be accomplished using immunohistochemistry assays, setting the basis for a more in-depth evaluation of H3K4me3 and H3K27me3 prognostic value.

From a biological standpoint, mutational analysis of the genes encoding for EZH2 and MLL2 and its correlation with H3K4me3 and H3K27me3 immunoexpression would be very interesting and eventually afford a functional basis for your findings. In the same vein, the identification of specific genes downregulated due to decreased H3K4me3 and/or increased H3K27me3 might also provide new targets for therapeutic intervention, and contribute to improve survival and quality of life of patients afflicted by DLCL.

A better understanding of the mechanism leading to B-cell lymphomagenesis was brought by recently discovered epigenetic alterations, although more studies are needed to better clarify their role and clinical relevance. Because several epigenetic alterations are also putative therapeutic targets, research in this field might be envisaged as promising and should be stimulated.



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## **ANNEXES**



## ANNEX 1

### Protocolos Epigenética

#### Imunohistoquímica



#### Imunohistoquímica (NovoLink Max Polymer Detection System)

1. Desparafinar e hidratar (4 minutos cada).
2. Lavar em água corrente (3 minutos) enquanto se faz o pré-aquecimento do tampão de recuperação (3 minutos).
3. Realizar a recuperação antigénica com tampão de recuperação Citrato ou EDTA a 1X (20min) no microondas.
4. Lavar em água corrente, seguida de água destilada (3 e 2 minutos, respectivamente).
5. Neutralizar a peroxidase endógena, com Peróxido de Hidrogénio a 0,6% (20 minutos).
6. Lavar em água corrente, seguida de água destilada (3 e 2 minutos, respectivamente).
7. Lavar em TBST (2 x 3 minutos).
8. Preparar a câmara húmida.
9. Marcar as lâminas com caneta hidrofóbica (este passo também pode ser realizado após o passo 6).
10. Incubar com **Protein Block** (5 minutos).
11. Lavar em TBST (2 x 3 minutos).
12. Incubar com anticorpo primário (o tempo e diluição apropriados).
13. Lavar em TBST (2 x 3 minutos).
14. Incubar com **Post Primary Block** (30 minutos).
15. Lavar em TBST (2 x 3 minutos).
16. Incubar com **Polymer** (30 minutos).
17. Lavar em TBST (3 minutos).
18. Lavar em PBS (3 minutos).
19. Incubar com DAB protegido da luz (7 minutos).
20. Lavar em água corrente.
21. Corar com Hematoxilina previamente filtrada (20 segundos).
22. Lavar em água morna, aproximadamente 1 minuto (diferenciação).
23. Desidratar, diafanizar e montar.

*O volume dos reagentes adicionado a cada lâmina deve ser o necessário de forma a cobrir todo o corte.*

## Protocolos Epigenética

### Imunohistoquímica



- Preparação de 1L de Citrato de Sódio pH6 1x
  - 100 mL de citrato de sódio pH6 10x
  - 900 mL de água destilada
  - Ou
  - 9 mL de citrato de sódio concentrado (solução comercial)
  - 960 mL de água destilada
- Preparação de 1L de EDTA pH8 1x
  - 100 mL de EDTA pH8 10x
  - 900 mL de água destilada
- Peróxido de Hidrogénio a 0,6%
  - 10 mL de Peróxido de Hidrogénio a 30%
  - 490 mL de água destilada
- Preparação de 1L de TBST 1x
  - 50 mL de TBS20x
  - 950 mL de água destilada
  - 1 mL de Tween
- Preparação DAB
  - 0,05 g de DAB
  - 100 mL de PBS 1x
  - 100  $\mu$ L Peróxido de Hidrogénio a 30%
  - A DAB deve ser preparada imediatamente antes de utilizar, devendo ser protegida a luz com papel de alumínio.